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EXPERIMENTAL STUDY

Effect of salvianolic acid A and C compatibility on inflammatory cytokines in rats with unilateral ureteral obstruction

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Abstract

OBJECTIVE: To investigate the effect of salvianolic acid A and C component molecules, which are involved in drug compatibility, on inflammatory cytokine expression that affects human chemokine ligand 5 (CCL5) and chemokine ligand 10 (CXCL10) levels in rats with unilateral ureteral obstruction (UUO).

METHODS: Fifty Sprague Dawley rats were randomly divided into five groups: normal, model, salvianolic acid A, salvianolic acid C and salvianolic acid A and C groups. The normal group was used as the control, and the other groups of rats had a UUO model established. The control group had free access to food and water, and the other groups received the corresponding drugs for 2 weeks. After the last administration, urine β_2-microglobulin (β_2-MG) and N-acetyl-β-D-glucosaminidase (NAG) levels were analyzed. After 24 h, all rats were sacrificed and the serum was analyzed for creatinine (Cr) and blood urea nitrogen (BUN) levels. Rat kidneys were removed, and CCL5 and CXCL10 inflammatory cytokine mRNA expression was measured using real-time fluorescent quantitative reverse transcription-polymerase chain reaction (RT-PCR). Kidney fibrosis was observed using hematoxylin-eosin (HE) staining and Masson trichrome staining.

RESULTS: In the salvianolic acid A and salvianolic acid C treatment groups, serum Cr and urine NAG levels were significantly lower than in the model group (both $P < 0.05$). In all treatment groups, urine β_2-MG levels were significantly lower than in the model group (all $P < 0.05$). Compared with model group, the pathological changes and collagen deposition improved to varying degrees (both $P < 0.05$). CCL5 and CXCL10 mRNA expression decreased to different degrees compared with the model group (both $P < 0.05$).

CONCLUSION: Salvianolic acid A and C are component molecules of drug compatibility, and they may protect renal function and improve tubular function and renal pathology to a certain degree in UUO. This improvement may be related to a reduction in inflammatory cytokines CCL5 and CXCL10 secretion in the UUO rat kidney.

Key words: Salvianolic acid; Ureteral obstruction; CCL5 protein, human; CXCL10 protein, human; Component compatibility

INTRODUCTION

Chronic kidney disease (CKD) is mainly characterized

by excessive deposition of extracellular matrix and fibrosis, causing decreased renal function. Because of its high morbidity and mortality, CKD has become a global public health problem. Renal fibrosis, including glomerulosclerosis and renal interstitial fibrosis (RIF), caused CKD and end-stage renal disease.[1] Related studies showed that RIF is more important than the glomerular lesions in the progression of CKD.[2] RIF is an important part of the clinical exacerbation process in chronic renal failure, and it is also is one of the main targets for Chinese medicine in prevention and treatment of CKD. Inflammatory cell infiltration is the basic pathology of CKD, and it is closely associated with RIF.[3] Inflammation induced by infiltration of inflammatory cells can cause tubular epithelial and fibroblast cells to secrete inflammatory chemokines and cause progressive tubulointerstitial damage and fibrosis, ultimately leading to end-stage renal disease by activating the leukocyte-mediated chemokine and chemokine receptor system. *In vitro* studies have demonstrated that fibroblast cells secrete several inflammatory chemokines in the process of renal fibrosis, including Chemokine (C-C Motif) Ligand 2 (CCL2), Chemokine (C-C Motif) Ligand 3 (CCL3), restructuring human chemokine ligand 5 (CCL5) and chemokine ligand 10 (CXCL10).[4] Related research has found that inhibiting tubulointerstitial leukocyte recruitment by blockade of chemokine action may mitigate chronic renal inflammation and subsequent fibrosis.[5] Thus, reducing inflammatory chemokine secretion may be a new target for prevention and treatment of renal fibrosis.

Danshen (*Radix Salviae Miltiorrhizae*) and Traditional Chinese Medicines containing a salvia decoction are widely used in the prevention and treatment of renal fibrosis.[6] A related study on water-soluble active components of Danshen (*Radix Salviae Miltiorrhizae*) demonstrated that water-soluble active components from Danshen (*Radix Salviae Miltiorrhizae*), including salvianolic acid A and C, have strong antioxidant activity and antithrombotic effect by scavenging oxygen free radicals and inhibiting lipid peroxidation reaction.[7] Kidney collateral stasis is a key pathogenesis of renal fibrosis. Therefore, salvianolic acid A and C component compatibility in renal fibrosis pathogenesis works through promoting blood circulation and removing blood stasis, cooling blood and eliminating the role of carbuncles. In this study, we selected salvianolic acid A and C in molecular medicine, and we used a UUO experimental model to study the effect of salvianolic acid A and C molecule compatibility on the inflammatory cytokines CCL5 and CXCL10. We established a new method of molecule drug compatibility in the prevention and treatment of CKD in the experimental study.

MATERIALS AND METHODS

Animals

Fifty healthy, specific pathogen-free male Sprague-Dawley rats, weighing 220-250 g (50-60 days old), were supplied and fed by the Medical Research Center of Guangdong Province of China (Certificate of quality No. SCXK [Yue] 2008-0002).

Modeling and grouping

A total of 50 rats were randomly divided into five groups using the random number table method, as follows: control group given saline lavage (n = 10), model group (n = 10), salvianolic acid A group (n = 10), salvianolic acid C group (n = 10) and salvianolic acid A+C group (n = 10). The UUO model was established through unilateral ureteral ligation. Firstly, after anesthesing rat and disinfectting the skin, away from the spine at approximately 1-1.5 cm, peeled subcutaneous fascia layer by layer and cut muscle to expose the renal pelvis and ureter. Then separated right ureter and cut it in the middle. Next, Finally, closed abdominal cavity, sterile dressing coverd.

Drugs and reagents

Salvianolic acid A and C was purchased from Ze Lang Pharmaceutical Company (Nanjing, China). Drugs were configured to the appropriate concentration with saline, and dosage was 12.50 $mg \cdot kg^{-1} \cdot d^{-1}$.[8] Rat β_2-microglobulin (β_2-MG) and N-acetyl-β-D-glucosaminidase (NAG) enzyme-linked immunosorbent assay (ELISA) kits were purchased from CUSABIO (Wuhan, China). The Masson staining kit was purchased from Jian Cheng Institute of Biotechnology (Nanjing, China). Trizol and AMV First Strand cDNA Synthesis Kit ABI and SybrGreen PCR Master Mix were purchased from Sangon Biotechnology Company (Shanghai, China).

Renal function assay

Twenty-four hours after the last intragastric administration, the rats in each group were anesthetized and their abdominal cavity was opened under sterile conditions to collect blood from the abdominal aorta. Serum was separated by centrifugation, and serum creatinine (Cr) and blood urea nitrogen (BUN) were measured using the enzymatic dynamic method.

Renal tubular function assay

After the last intragastric administration, rats were placed in metabolic cages to collect urine. Urine β_2-MG and NAG were tested using ELISA kits, according to the manufacturer's instructions.

Renal pathology observation and score

Half the kidney tissue was fixed in 4% paraformaldehyde, dehydrated in ethanol, embedded in paraffin and sliced to a thickness of approximately 3 μm for routine hematoxylin-eosin (HE) staining and Masson trichrome staining. Pathological changes in renal tissues were observed under light microscopy. HE staining of tubulointerstitial injury were evaluated using semi-quantitative scoring according to the Banff criteria:[9] (a) no

tubular dilatation, inflammation, atrophy or necrosis was scored as 0; (b) mild pathological changes and tubulointerstitial lesions less than 25% was scored as 1 point; (c) moderate pathological changes with tubulointerstitial lesions ranging from 26%-50% was scored as 2 points; and (d) severe pathology changes and greater than 50% tubulointerstitial lesions was scored as 3 points. In each slice, 10 consecutive non-overlapping high power (× 400) fields were counted, and the average was calculated to compare the extent of tubulointerstitial injury among the groups. As described above. The average score of each slice was calculated to compare the degree of RIF among all groups. Photographs of HE and Masson staining were taken using an inverted fluorescence microscope (IX71, Olympus Co., Tokyo, Japan).

Real-time fluorescent quantitative reverse transcription-polymerase chain reaction microarray

RNA was prepared using Trizol, and its yield and quality were assessed using UV absorbance and denaturing agarose gel electrophoresis. First strand cDNA was synthesized by Reverse Transcriptase, and cDNA samples were diluted and loaded for analysis using 96-well real-time PCR arrays (ABI Co., Carlsbad, CA, USA). All assays were performed according to the manufacturer's protocols. β-actin was used as an internal control gene during the experiment. The real-time amplification curve and the dissociation curve of each gene were then obtained. The cycle threshold (Ct) values were obtained, and the $2^{-\Delta\Delta Ct}$ Method[10] was used to calculate the relative expression of target genes (Table 1).

Statistical analysis

All data were analyzed using SPSS 16.0 (SPSS, Chicago, IL, USA) software. Quantitative data are expressed as he mean ± standard deviation ($\bar{x} \pm s$). The single factor analysis of variance for a completely randomized design was used to compare parameters among groups. The least significant difference method and Tamhane's T2 test were used to compare parameters between groups. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of salvianolic acid A and C compatibility on renal function

Serum Cr and BUN levels showed that renal function was significantly damaged in UUO rats compared with the control group ($P < 0.05$). In the salvianolic acid A and salvianolic acid C treatment groups, serum Cr levels were significantly reduced compared with control group (both $P < 0.05$), and there was no significant difference between the other treatment groups and the model group ($P > 0.05$). There was no significant difference in serum BUN levels between any treatment groups and the model group ($P > 0.05$; Table 2).

Effect of salvianolic acid A and C compatibility on renal tubular function

β_2-MG and NAG levels in UUO rats were significantly higher than those in the control group ($P < 0.05$). In all treatment groups, β_2-MG levels were significantly lower than the model group (all $P < 0.05$), and there were no significant differences among the treatment groups (all $P < 0.05$). In the salvianolic acid A and salvianolic acid C groups, NAG levels were significantly reduced (both $P < 0.05$), and in the salvianolic acid C group, the NAG level was significantly lower than the salvianolic acid A+C group ($P < 0.05$; Table 3).

Table 1 Gene primer sequences

Gene	GenBank access number	Forward primer (5′-3′)	Reverse primer (5′-3′)
CCL5	NM_031116.3	GTGCCAACCCAGAGAAGAAGT	GAGCAAGCAATGACAGGAAAG
CXCL10	NM_139089.1	AAAGCGGTGAGCCAAAGAA	GGAGAAACAGGGACAGTTAGGAC
β-actin	NM_031144.3	CGTAAAGACCTCTATGCCAACA	AGCCACCAATCCACACAGAG

Notes: CCL5: restructuring human chemokine ligand 5; CXCL10: chemokine ligand 10.

Table 2 Effect of salvianolic acid A and C compatibility on serum Cr and BUN levels in UUO rats ($\bar{x} \pm s$)

Group	*n*	Cr (μmol/L)	BUN (mmol/L)
Control	7	28.2±3.0	7.8±0.5
UUO	7	44.1±16.0[a]	11.4±1.8[a]
Salvianolic acid A	7	32.4±6.8[b]	9.3±0.8[a]
Salvianolic acid C	7	30.8±7.4[b]	12.3±2.2[a]
Salvianolic acid A and C	7	38.0±7.1	9.5±1.4

Notes: control group was treated with physiological saline for 2 weeks. UUO group was treated with physiological saline for 2 weeks. Salvianolic acid A group was treated with salvianolic acid A 12.50 mL/kg for 2 weeks. Salvianolic acid C group was treated with salvianolic acid C 12.50 mL/kg for 2 weeks. Salvianolic acid A and C group was treated with 6.25 mL/kg salvianolic acid A and 6.25 mL/kg salvianolic acid C for 2 weeks. Cr: creatinine; BUN: blood urea nitrogen; UUO: unilateral ureteral obstruction. [a]$P < 0.05$, compared with the control group; [b]$P < 0.05$, compared with the UUO group.

Effect of salvianolic acid A and C compatibility on renal pathology

In UUO rats, interstitial tubules were severely damaged and there was significant collagen deposition compared with the control group ($P < 0.05$). In all treatment groups, the pathological changes and collagen deposition improved to varying degrees (all $P < 0.05$). In the salvianolic acid A group and salvianolic acid A+C group, the scoring with HE and Masson staining was lower than the salvianolic acid C group (both $P < 0.05$). In the salvianolic acid A+C group, the scoring with Masson staining was lower than the salvianolic acid A group ($P < 0.05$) (Table 4, Figures 1 and 2).

Results of fluorescent quantitative RT-PCR microarray

Microarray analysis showed that CCL5 and CXCL10 gene expression levels in UUO rats were significantly higher than that in the control group ($P < 0.05$). The treatment groups had varying degrees of reduced expression compared with the model group (all $P < 0.05$). In the salvianolic acid A+C group, CCL5 mRNA expression decreased more significantly than the model

Table 3 Effect of salvianolic acid A and C compatibility on β_2-MG and NAG in UUO rats (μmol/L, $\bar{x} \pm s$)

Group	*n*	β_2-MG	NAG
Control	6	0.167±0.003	1.137±0.109
UUO	6	0.182±0.005[a]	2.768±0.516[a]
Salvianolic acid A	6	0.174±0.006[ab]	2.081±0.482[ab]
Salvianolic acid C	6	0.176±0.005[ab]	1.667±0.544[ab]
Salvianolic acid A and C	6	0.174±0.004[ab]	2.347±0.343[ac]

Notes: control group was treated with physiological saline for 2 weeks. UUO group was treated with physiological saline for 2 weeks. Salvianolic acid A group was treated with salvianolic acid A 12.50 mL/kg for 2 weeks. Salvianolic acid C group was treated with salvianolic acid C 12.50 mL/kg for 2 weeks. Salvianolic acid A and C group was treated with 6.25 mL/kg salvianolic acid A and 6.25 mL/kg salvianolic acid C for 2 weeks. β_2-MG: β_2-Microglobulin; NAG: N-acetyl-β-D-glucosaminidase; UUO: unilateral ureteral obstruction. [a]$P < 0.05$, compared with the control group; [b]$P < 0.05$, compared with the UUO group; [c]$P < 0.05$, compared with salvianolic acid C group.

Table 4 Renal pathological evaluation of salvianolic acid A and C compatibility in UUO rat kidney tissue ($\bar{x} \pm s$)

Group	*n*	HE score	Masson score
Control	6	0.05±0.06	0.03±0.05
UUO	6	2.65±0.10[a]	2.65±0.10[a]
Salvianolic acid A	6	1.32±0.18[abc]	1.72±0.13[abc]
Salvianolic acid C	6	2.35±0.19[ab]	1.97±0.15[ab]
Salvianolic acid A and C	6	1.18±0.18[abc]	1.50±0.13[abcd]

Notes: control group was treated with physiological saline for 2 weeks. UUO group was treated with physiological saline for 2 weeks. Salvianolic acid A group was treated with salvianolic acid A 12.50 mL/kg for 2 weeks. Salvianolic acid C group was treated with salvianolic acid C 12.50 mL/kg for 2 weeks. Salvianolic acid A and C group was treated with 6.25 mL/kg salvianolic acid A and 6.25 mL/kg salvianolic acid C for 2 weeks. UUO: unilateral ureteral obstruction. [a]$P < 0.05$, compared with the control group; [b]$P < 0.05$, compared with the UUO group; [c]$P < 0.05$, compared with salvianolic acid C group; [d]$P < 0.05$, compared with salvianolic acid A group.

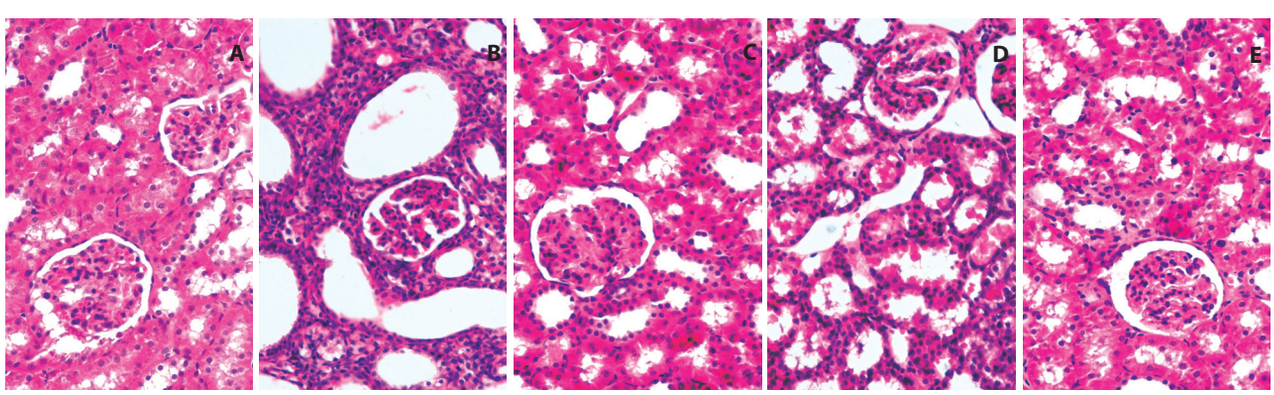

Figure 1 Pathological changes in the rat kidney in the different groups (HE staining, ×400)

A: Control group; B: UUO group; C: salvianolic acid A group; D: salvianolic acid C group; E: salvianolic acid A and C group. Control group was treated with physiological saline for 2 weeks. UUO group was treated with physiological saline for 2 weeks. Salvianolic acid A group was treated with salvianolic acid A 12.50 mL/kg for 2 weeks. Salvianolic acid C group was treated with salvianolic acid C 12.50 mL/kg for 2 weeks. Salvianolic acid A and C group was treated with 6.25 mL/kg salvianolic acid A and 6.25 mL/kg salvianolic acid C for 2 weeks. HE: hematoxylin-eosin; UUO: unilateral ureteral obstruction.

group ($P < 0.05$). In the salvianolic acid A group and salvianolic acid A + C group, CXCL10 expression was lower than the salvianolic acid C group (both $P < 0.05$). The β-actin amplification curve, CCL5 and CXCL10 had a good amplified signal, and the dissociation curve for each gene had only a single peak, indicating the specificity of amplification (Table 5).

DISCUSSION

Serum Cr and BUN have been widely used as indicators of renal function. Serum Cr and BUN levels were significantly elevated in UUO rats compared with the control rats, confirming that the UUO model of RIF had been successfully generated. In the salvianolic acid A and salvianolic acid C treatment groups, serum Cr levels were significantly decreased compared with the model group. However, there was no significant dose-response relationship for the serum BUN levels between any of the treatment groups and the model group. This showed that salvianolic acid A and C could protect renal function in UUO rats.

β_2-MG is a small-molecule immunoglobulin that is produced by lymphocytes, platelets and polymorphonuclear leukocytes. It has constant rate of synthesis and release under normal circumstances, and the content in normal urine is low. Almost all β_2-MG can be free-filtered from the glomerulus, and more than 99% is reabsorbed by the proximal tubule, then transported to the lysosomes and resolved into amino acids. When renal function is impaired, the glomerular filtration rate is reduced and clearing β_2-MG is difficult, resulting in increasing urine β_2-MG levels, which indicates a reduction in tubular reabsorption function. Therefore, urine β_2-MG is considered to be a classic marker protein for tubules that can directly reflect tubular function.[11,12] NAG is a 140-KD lysosome that is mostly stored in the nephron proximal convoluted tubule cells. Its half-life in plasma is approximately 5 min, and plasma NAG can't through the glomerulus. The increase in urine NAG activation suggests that the proximal tubular epithelial cell dysfunction may be a result of renal toxicity, leading to lysosome rupture and the release NAG. NAG is an another sensitive early indicator of renal tubular injury.[13] Our results showed that urine β_2-MG and NAG levels were significantly increased in UUO rats compared with normal rats, and β_2-MG levels were significantly decreased in all treatment groups. NAG levels were significantly reduced in the salvianolic acid A and salvianolic acid C groups. This indicated that salvianolic acid A and C compatibility could improve renal tubular function to a certain degree in UUO rats.

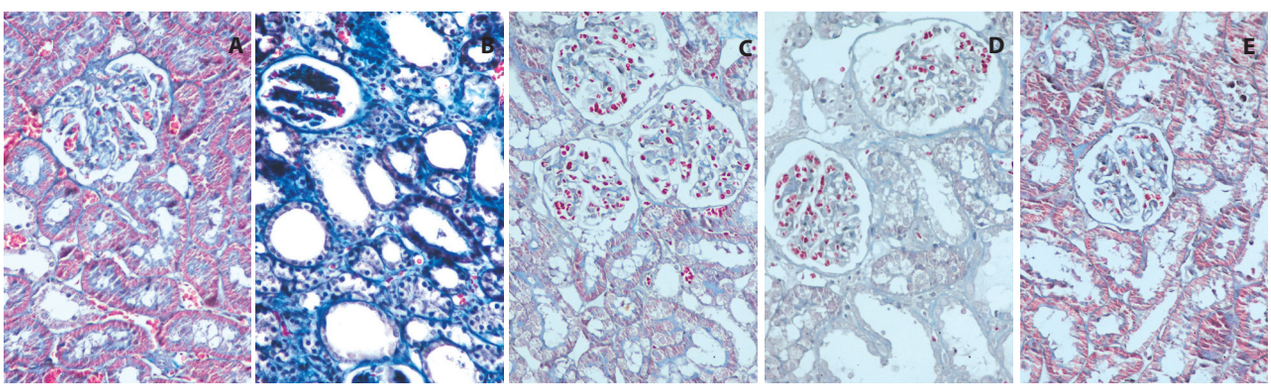

Figure 2 Pathological changes in the rat kidney in the different groups (Masson staining, ×400)
A: control group; B: UUO group; C: salvianolic acid A group; D: salvianolic acid C group; E: salvianolic acid A and C group. Control group was treated with physiological saline for 2 weeks. UUO group was treated with physiological saline for 2 weeks. Salvianolic acid A group was treated with salvianolic acid A 12.50 mL/kg for 2 weeks. Salvianolic acid C group was treated with salvianolic acid C 12.50 mL/kg for 2 weeks. Salvianolic acid A and C group was treated with 6.25 mL/kg salvianolic acid A and 6.25 mL/kg salvianolic acid C for 2 weeks. UUO: unilateral ureteral obstruction.

Table 5 Effect of salvianolic acid A and C on CCL5 and CXCL10 expression levels in UUO rats ($\bar{x} \pm s$)

Group	*n*	CCL5	CXCL10
Control	3	1.51±0.45	1.10±0.18
UUO	3	11.15±0.80[a]	6.31±0.97[a]
Salvianolic Acid A	3	6.20±0.43[abc]	2.41±0.56[abc]
Salvianolic Acid C	3	8.12±0.83[ab]	3.67±0.84[ab]
Salvianolic Acid A and C	3	5.12±0.16[abcd]	2.33±0.48[abc]

Notes: control group was treated with physiological saline for 2 weeks. UUO group was treated with physiological saline for 2 weeks. Salvianolic acid A group was treated with salvianolic acid A 12.50 mL/kg for 2 weeks. Salvianolic acid C group was treated with salvianolic acid C 12.50 mL/kg for 2 weeks. Salvianolic acid A and C group was treated with 6.25 mL/kg salvianolic acid A and 6.25 mL/kg salvianolic acid C for 2 weeks. CCL5: restructuring human chemokine ligand 5; CXCL10: chemokine ligand 10; UUO: unilateral ureteral obstruction. Compared with the control group, [a]$P < 0.05$; compared with the UUO group, [b]$P < 0.05$; compared with salvianolic acid C group, [c]$P < 0.05$; compared with salvianolic acid A group, [d]$P < 0.05$.

We found that kidneys in UUO model rats were significantly larger than those from normal rats, the renal pelvis and calyces were dilated into cysts that contained urine, the renal parenchymal showed atrophy and thinning, and the cortex and medulla boundaries were indistinct. Under light microscopy, histopathological changes in the renal tissue indicated that the UUO model had been successfully established. In all treatment groups, the histopathological changes were significantly improved compared with the model group. In the salvianolic acid A group and salvianolic acid A + C group, HE and Masson scores were lower than in the salvianolic acid C group. In the salvianolic acid A + C group, Masson scores were lower than the salvianolic acid A group. Thus, salvianolic acid A and C compatibility could improve renal pathology to a certain degree in UUO rats, and there were some dose-response effects.

Previous studies showed that renal interstitial inflammation injury is the fundamental cause of fibrosis.[14,15] This process is mediated in a paracrine manner because inflammatory cells can activate fibroblasts and tubular epithelial cells to secrete pro-fibrotic factors and thereby promote RIF.[16,17] Inflammatory chemokines are pro-fibrotic factors with a relatively low molecular weight (8-10 Kd), which are secreted by different types of cells. They have a chemotaxis effect on various kinds of leukocyte subclasses such as neutrophils, monocytes and lymphocytes,[18,19] and they are the critical factors that initiate the renal interstitial inflammatory cascade reaction.[18,20] To date, inflammatory chemokines have been grouped into four families: CXC, CC, CX3C and XC. The main families are CXC and CC. These inflammatory chemokines bind to the seven transmembrane G-protein-coupled receptor families; CCL5 belongs to the CC family, while CXCL10 belongs to the CXC family.[21] Many factors involved in kidney damage can induce tubular epithelial cells expression of inflammatory chemokines, thereby promoting inflammatory cell infiltration, and gradually forming RIF that is promoted by interstitial inflammation.[22] In this research, we found that CCL5 levels and CXCL10 gene expression in UUO rats were significantly greater than in the control group; the treatment group had a greater different amount of reduction than the model group. This suggests that the secretion of inflammatory chemokines increased in UUO rats, and that these inflammatory cytokines are involved in the development and progression of renal fibrosis. We also showed that salvianolic acid A and C compatibility could interfere with UUO in the rat kidney through CCL5 and CXCL10 secretion.

In conclusion, our study demonstrated that the effect of molecule drug compatibility on the renal fibrosis mechanism had several targets. These findings provide a scientific rationale for the basis of using salvianolic acid A and C to treat patients with CKD. Further research is necessary to obtain a full understanding of the appropriate way to modulate renal fibrosis.

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